OPEN ACCESS

University of Dundee

University of Dundee

Olaparib, monotherapy or with ionizing radiation, exacerbates DNA damage in normal tissues

McMahon, Michael; Frangova, Tania G.; Henderson, Colin J.; Wolf, C. Roland

Published in:
Molecular Cancer Research

DOI:
10.1158/1541-7786.MCR-16-0108

Publication date:
2016

Document Version
Peer reviewed version

Link to publication in Discovery Research Portal

Citation for published version (APA):
McMahon, M., Frangova, T. G., Henderson, C. J., & Wolf, C. R. (2016). Olaparib, monotherapy or with ionizing radiation, exacerbates DNA damage in normal tissues: insights from a new p21 reporter mouse. *Molecular Cancer Research*, *14*(12), 1195-1203. https://doi.org/10.1158/1541-7786.MCR-16-0108

General rights
Copyright and moral rights for the publications made accessible in Discovery Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from Discovery Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the public portal.

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 04. May. 2023

SUPPORTING INFORMATION

Appendix: Expression of β-galactosidase in control and irradiated reporter mice.

Unfortunately, there is no single gold-standard resource describing p21 protein expression at single cell resolution against which we can benchmark our β-galactosidase staining patterns. In fact, there is a surprising paucity of p21 immuno-histochemical data in the literature, for which reason we have occasionally inferred the likely anatomical location of mouse p21 protein from *in situ* hybridisation data or the location of the orthologous human protein. Although the data contained in the papers we reference are partial, occasionally contradictory, and sometimes hard to interpret, in aggregate they support the conclusion that β-galactosidase activity is a *bone fide* reporter for p21 protein.

Brain (Fig S3). β-galactosidase expression in this anatomically complex organ best displays the resolving power of our technology. In the cerebellum, only Purkinje cells were positive, whereas in the cortex, pyramidal neurons in regions responsible for sensation, such as audition (DCN) and olfaction (AONm, PIR and OUT) were highlighted. Finally, we found that cells present within the subgranular zone (SGZ) of the dentate gyrus and ependymal cells in the subventricular zone were reporter-positive also. As these are the only two known sources of neural precursor cells (NPC) in the adult mouse, these data suggest that p21 plays a general role in restricting the proliferation of adult NPCs until necessary.

Consistent with our contention that β-galactosidase faithfully marks p21 expression, sporadic reports have previously located p21 in Purkinje cells in the cerebellum, neurons within the olfactory bulb of the cortex, and NPCs within the subgranular zone (SGZ) of the dentate gyrus (1–3). Impressively, our data capture all of the above-mentioned features – and more – in a single experiment, albeit we find β-galactosidase positive cells scattered throughout the

dentate gyrus rather than restricted to the SGZ. This might reflect the reduced levels of p21 in reporter mice.

Gastrointestinal (GI) tract (Fig 2; for whole-organ image of large intestine, please see Fig S7): A complex pattern was observed throughout the GI tract. A high level of β-galactosidase activity was observed in the keratinized, squamous epithelium of the forestomach. In the glandular stomach, there was a striking demarcation in the fundic mucosa between the parietal and chief cells at the base of the glands (β-galactosidase positive) and the foveolar mucous-secreting neck cells (β-galactosidase negative). There was also a marked contrast between the mucosae of the small and large intestine. β-galactosidase positive cells were predominantly found in villous epithelial cells with a trend towards higher β-galactosidase expression in those cells immediately adjacent to the crypt rather than those at the villus tip. Few cells stained positive within the crypt epithelium but, strikingly, these were at the very base and may represent either Paneth cells or stem cells. In contrast, staining was largely confined to terminally-differentiated, apical epithelial cells in the large intestine and only a scattering of positive cells were observed deeper within the crypts. In both small and large intestines, staining was generally absent from the *lamina propria*, but the smooth muscle cells of the *muscularis externae* and *muscularis mucosae* were frequently positive. Finally, in response to irradiation, β-galactosidase was highly induced in epithelial cells throughout the GI tract. This was particularly pronounced in the small- and large intestines where essentially all epithelial cells became β-galacosidase positive.

We could not find data on the spatial distribution of p21 across the mouse gastrointestinal tract. However, consistent with our data, El-Deiry and colleagues reported a striking compartmentalization of p21 expression throughout normal gastrointestinal tract of humans

(4). They found that p21 was expressed mainly in the terminally-differentiated epithelial cells of the large intestine. Also in agreement with us was their suggestion that the protein was expressed primarily in the post-proliferative cells of the small intestine. Finally, we noted that isolated cells near the base of the crypts also expressed β-galactosidase, a finding that chimes with the report by George and co-workers of sporadic p21-positive cells in the small intestinal crypts of mice (5).

Lung (Fig S4; for whole-organ image of lung, please see Fig S7): the β-galactosidase signal in lung was located predominantly in bronchial epithelial and smooth muscle cells lining pulmonary veins; a scattering of positive cells were observed within the alveolus, but their identity is uncertain at present. After irradiation, essentially all cells in the alveolar parenchyma were found to be positive.

Consistent with our findings, Bouvard and colleagues reported p21 expression in all bronchial epithelial cells and alveolar cells 6 h following IR(6). Staining was much less intense in control lungs.

Kidney (Fig S4): Within the cortex, we observed infrequent positive cells in glomeruli, which were comprised mainly of epithelial cells in the Bowman's capsule but some podocytes also. Positive interstitial cells could also be observed in the kidney medulla. The number of positive cells increased after irradiation. Although not shown in **Fig S4**, β-galactosidase positive cells become more frequent as the medulla turns into the papilla.

Regarding prior data, Megyesi et al. reported p21 protein to be restricted to a sparse population of tubular epithelial cells (7), as do we. However, these authors failed to detect p21 in glomeruli, which conflicts with our finding β-galactosidase positive cells within these structures. The reason for this discrepancy remains unresolved. They did not comment on

p21 expression in the medulla or papilla. Finally, Bouvard and colleagues found that p21 was expressed in some glomerular and tubular cells after exposure of mice to ionizing radiation (8), as did we.

Liver (Fig S4; for whole-organ image of liver, please see Fig S7): We identified β-galactosidase positive cells in the biliary epithelia and cells lining the portal vein. The signal intensifies over time post-irradiation and expands from the hepatic artery towards the central vein, essentially following the path of the bloodstream.

Induction of *p21* mRNA in mouse liver after exposure to ionizing radiation has been described previously, but zonation was not commented upon (9).

Heart (Fig S5): A substantial number of cardiomyocytes expressed p21, as established by β-galactosidase positive staining. The signal was somewhat increased 24 h after exposure to 4 Gy of IR.

Bouvard and colleagues reported a low p21 signal in cardiomyocytes, that was increased after irradiation (8).

Lymphoid tissues (Fig S5): Within the spleen, a limited number of positive cells were observed. These generally were endothelial cells surrounding vascular channels. A greater number of positive cells were observed in the thymus than in the spleen, and were largely restricted to the medulla. Based on size, these appear to be epithelial or dendritic in nature, rather than lymphoid. There was a dramatic increase in the number of β-galactosidase positive cells in the spleen and thymus 24 h post-irradiation. There was some evidence for positivity amongst aggregated lymphoid cells that were not observed in tissues from control animals.

Regarding previous data in the literature, there are no reports on p21 expression in normal murine spleen or thymus that we are aware of. However, one report found that human p21 is normally found expressed predominantly in Hassall's corpuscles (10), a structure in the thymic medulla comprised of large epithelial reticular cells. Although Hassall's corpuscles are absent in mice, our β-galactosidase positive cells have characteristics similar to these p21-positive cells, which they may perhaps be equivalent to.

Female reproductive tract (**Fig S6).** A complex staining pattern is evident throughout this organ system. In control ovaries, we found that the *Corpus lutea* were β-galactosidase-positive, with the follicles being negative. The muscle cells in the surrounding *bursa* are invariably positive also. Within the uterine horn, the inner and outer muscle layers of the myometrium are positive whereas endometrial cells are negative. After exposure to IR, there were significant changes in the pattern of β-galactosidase expression throughout the female reproductive tract. Ovarian follicles were now found to be positive as were endometrial cells – both epithelial and stromal – in the uterus.

There is no pre-existing data on the spatial distribution of p21 protein throughout this organ system against which to benchmark our data. However, there is one report in the literature describing p21 mRNA being expressed in Corpus Luteum (11), which supports our findings.

Materials and Methods

Chemicals and γ-irradiation: Olaparib (Selleck Chemicals) at a purity of greater than 99.8% was prepared at 100 mg/ml in DMSO. Prior to *p.o.* administration to mice, it was diluted to 7.5 mg/ml in PBS containing 10% (w/v) 2-hydroxy-propyl-β-cyclodextrin (Sigma). Clinical grade cisplatin at 1 mg/ml in 0.9% saline solution (Accord Healthcare) was used for these

experiments, with 0.9% NaCl as vehicle control (BI Braun). Clinical grade etoposide at 20 mg/ml was also obtained from Accord Healthcare and PBS was used as vehicle control. Mice were γ-irradiated in an Oris IBL 637 Cesium-137 irradiator (3 min/Gy).

Olaparib pharmacokinetics: Triplicate female adult wild-type mice were dosed *p.o*. with olaparib (dissolved in PBS containing 10% (v/v) dimethyl sulfoxide and 10% (w/v) 2-hydroxy-propyl-β-cyclodextrin) at 10, 50 or 100 mg/kg. Whole blood (10 µl) was taken from the tail vein at intervals after drug administration (10, 20, 40, 60, 120, 180, 240, 360 and 480 min) and transferred into a tube containing heparin (10 µl, 15 IU/ml). Samples were stored at -20 ˚C until analysed. Measurement of Olaparib in whole-blood was performed by Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS). Briefly, 50 µl of water containing 0.2 µg/ml Pazopanib (internal standard) and 500 µl of ethyl acetate was added to each sample and mixed thoroughly. After centrifugation (16,000 x *g*, 10 min), the organic phase was transferred to a fresh tube, dried-down using an Eppendorf Concentrator Plus (Eppendorf, Stevenage, UK), and the solute was resuspended in 60 µl of methanol before adding a further 20 µl of de-ionized H_2O. Olaparib and pazopanib were resolved on a Kinetex C18 column, (50 x 4.6 mm, 2.6 µm column (Phenomenex, Macclesfield, UK)) using a gradient of 100% of 20 mM ammonium formate to 100% of 0.1% (v/v) formic acid in acetonitrile with positive ion detection on a Waters 2795 Alliance HT and Quattro Micro mass spectrometry system (Micromass, Manchester, United Kingdom). Concentration of parent drug was calculated using an authentic standard curve prepared and analysed in the same manner as the pharmacokinetic samples. Pharmacokinetic parameters were determined using WinNonLin software, v3.1 (Pharsight (St Louis, MO, USA)). A non-compartmental model was used to calculate area under the curve ($AUC_{0\text{-}8h}$ and AUC_{inf}), terminal half-life, maximum plasma concentration (C_{max}), and clearance (Cl).

Immunoblots: Whole-cell lysates were prepared from flash-frozen organs as follows. Briefly, the organ was pulverised under liquid nitrogen with a mortar and pestle. The powder was added to Laemmli sample buffer (13 mM Tris (pH 6.8), 11% (v/v) glycerol, 0.44% (w/v) SDS, 0.02% (w/v) bromophenol blue, 1.1% (v/v) 2-mercaptoethanol) supplemented with Complete EDTA-free protease inhibitors (Millipore) and HALT phosphatase inhibitors (Thermo Scientific) and vortexed vigorously. After 30 min on ice, the resulting lysate was sonicated to reduce viscosity and protein concentrations were determined using the 'Microplate BCA Protein Assay Kit – Reducing Agent Compatible' from Thermo Scientific, according to the manufacturer's instructions. SDS/polyacrylamide-gel electrophoresis and immunoblotting were carried out as previously described (12). Antibodies used included mouse monoclonal antibodies raised against GAPDH (clone GAPDH_71.1 (Sigma)) β-gal (Promega), firefly luciferase (clone luci17 (Abcam)), and p21 (clone SX118 (BD Pharmingen)). The affinity-purified rabbit anti-p53 polyclonal antibodies (CM5) have previously been described (13).

Relative quantification of mRNA species: This was carried out by Taqman® chemistry. Total RNA was isolated from cells using the RNeasy Kit (Qiagen), according to the manufacturer's instructions. Approximately 1.0 μg of total RNA was reverse-transcribed to cDNA using the Qantitect kit (Qiagen), according to the manufacturer's instructions. The PCR mixes were prepared by mixing 1.5 μl of cDNA with 1 μl of TaqMan probe set, 10 μl of Universal PCR Master Mix (PerkinElmer Applied Biosystems) and 7.5 μl of MilliQ grade water. For the real-time PCR analysis, the following pre-designed TaqMan probe sets in solution were used: Mm00432448_m1 (p21); Hs03003631 (18s ribosomal RNA) (all from PerkinElmer Applied Biosystems). Data acquisition and analysis utilised the ABI PRISM® 7700 sequence detection system (PerkinElmer Applied Biosystems). The relative gene expression levels in different

samples were calculated using the Comparative C_T Method as outlined in the ABI PRISM® 7700 Sequence Detection System User Bulletin #2. The expression of 18s rRNA was used as the internal control.

Clinical chemistry: Terminal bleeds were collected in heparinized blood collection tubes (Sarstedt). Clinical chemistry assays were performed blind at the Clinical Pathology laboratory, MRC Harwell (http://www.har.mrc.ac.uk/services/pathology/clinical-chemistry).

References

1. Legrier ME, Ducray a, Propper a, Kastner a (2001) Region-specific expression of cell cycle inhibitors in the adult brain. *Neuroreport* 12(14):3127–3131.
2. Pechnick RN, Zonis S, Wawrowsky K, Pourmorady J, Chesnokova V (2008) p21Cip1 restricts neuronal proliferation in the subgranular zone of the dentate gyrus of the hippocampus. *Proc Natl Acad Sci U S A* 105:1358–1363.
3. Parker SB, et al. (1995) p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells. *Science* 267(February):1024–1027.
4. El-Deiry WS, et al. (1995) Topological Control of p21WAF1/CIPExpression in Normal and Neoplastic Tissues. *Cancer Res* 55:2910–2919.
5. George RJ, et al. (2009) Loss of p21 Waf1 / Cip1 / Sdi1 enhances intestinal stem cell survival following radiation injury. *Am J Physiol Gastrointest Liver Physiol* 296:245–254.
6. Bouvard V, et al. (2000) Tissue and cell-specific expression of the p53-target genes: bax, fas, mdm2 and waf1/p21, before and following ionising irradiation in mice. *Oncogene* 19(5):649–60.
7. Megyesi J, Andrade L, Vieira J.M. J, Safirstein RL, Price PM (2001) Positive effect of the induction of p21WAF1/CIP1 on the course of ischemic acute renal failure. *Kidney Int* 60(6):2164–2172.
8. Bouvard V, et al. (2000) Tissue and cell-specific expression of the p53-target genes: bax, fas, mdm2 and waf1/p21, before and following ionising irradiation in mice. *Oncogene* 19:649–660.
9. Fei P, Bernhard EJ, El-Deiry WS (2002) Tissue-specific induction of p53 targets in vivo. *Cancer Res* 62(24):7316–7327.
10. Kanavaros P, Stefanaki K, Rontogianni D (2001) Immunohistochemical expression of p53, p21/WAF1, Rb, p16, cyclin D1, p27, Ki67, cyclin A, cyclin B1, bcl2, bax and bak proteins and apoptotic index in normal thymus. *Histol Histopathol* 16(4):1005–1012.

11. Robker RL, Richards JS (1998) Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1. *Mol Endocrinol* 12(7):924–940.
12. McMahon M, Itoh K, Yamamoto M, Hayes JD (2003) Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J Biol Chem* 278(24):21592–21600.
13. Midgley CA , Owens B , Briscoe CV , Thomas DB , Lane DP HP (1995) Coupling between gamma irradiation , p53 induction and the apoptotic response depends upon cell type in vivo. *J Cell Sci* 108(July 2016):1843–1848.

SI Figures

Figure S1: Quantitative analysis of the immunoblots of Fig 1B. Densitometric data were extracted from tiff files using the Gel Analysis method outlined in the ImageJ documentation: Gel Analysis. P21, p53, β-gal and Luc intensity data were normalized based on the corresponding GAPDH data. The normalized p21 and p53 intensity data were transformed so that the value in wild-type mice was set to 1. The normalized β-gal and Luc data were transformed so that the value in homozygous reporter mice was set to 1.

Figure S2: Quantitative analysis of the immunoblots of Fig 1C. Densitometric data were extracted from tiff files using the Gel Analysis method outlined in the ImageJ documentation: Gel Analysis. P21, p53, β-gal and Luc intensity data were normalized based on the corresponding GAPDH data. The normalized intensity data were transformed so that the value at 0 h was set to 1.

Figure S3: β-gal staining of brain sections from an untreated p21 reporter mouse. Brain sections were prepared and stained as described in Materials and Methods. Scale-bar = 1000 μm (main images) or 100 μm (insets). Abbreviations: CTX, cortex; AONm, anterior olfactory nucleus, medialis; PIR, piriform cortex; OTU, olfactory tubercle; LV, lateral ventricle; HPC,

hippocampus proper; DG, dentate gyrus; S, subiculum; DCN, dorsal cochlear nucleus; CP, choroid plexus; PCL, purkinje cell layer.

Figure S4: β-gal staining of lung, liver, and kidney sections from untreated and irradiated p21 reporter mice. Mice were sham-irradiated (-IR) or exposed to a single dose of 4 Gy IR (+IR) and sacrificed 24 h later. Representative images are shown. Scale-bar = 100 μm except liver (50 μm). PV, pulmonary vein; B, bronchiole; P, portal vein; C, central vein; G, glomerule; T, tubule. Arrow (podocytes), arrowhead (Bowman's capsule).

Figure S5: β-gal staining of spleen, thymus, and heart sections from untreated and irradiated p21 reporter mice. Mice were sham-irradiated (-IR) or exposed to a single dose of 4 Gy IR (+IR) and sacrificed 24 h later. Representative images are shown. Scale-bar = 100 μm.

Figure S6: β-gal staining of uterus sections from untreated and irradiated p21 reporter mice. or 500 μm (blow-ups). B, Ovarian bursa; CL, *Corpus luteum*; E, endometrium; M, myometrium. Arrowheads point to follicles.

Figure S7: β-gal staining of whole lung, large intestine and liver sections from untreated and irradiated p21 reporter mice. Mice were sham-irradiated (CONTROL) or exposed to a single dose of 4 Gy IR (IR) and sacrificed 24 h later. Representative images of *in situ* β-gal staining of some whole organs are presented. Scale-bar = 1000 μm.

Figure S8: B-gal staining is localized to the nucleus of cells. *In situ* β-gal staining of a liver section from a control mouse is shown. The β-gal signal is located in the nucleus of individual hepatocytes. Nuclear Fast Red was used as a counterstain. Scale-bar = 50 μm.

Figure S9: Kidney and liver function tests on plasma from cisplatin-treated reporter mice. Urea levels, and alanine aminotransferase (ALT) and aspartate aminotransferase AST)

activities were determined in plasma samples from reporter mice ($\bar{x}$ ± SD, *n* = 3 except 10 mg/kg where *n* = 2).

Figure S10: β-gal staining of various organs from control and etoposide-treated p21 reporter mice. Mice were treated with vehicle (PBS) or 40 mg/kg etoposide and sacrificed 24 h later. Representative images are shown. Scale-bar = 100 μm.

Figure S11: Bioluminescence from irradiated reporter mice. A magnified view of the mice displayed in Fig 4.

Figure S12: Visceral organ bioluminescence observed by invasive imaging. Triplicate mice were treated with 40 mg/kg etoposide and non-invasively imaged 24 h later alongside duplicate vehicle (PBS)-treated mice. Mice were then sacrificed, their abdomens cut open to reveal the underlying viscera, and re-imaged.

Figure S13: Pharmacokinetic parameters for olaparib in wild-type mice are linearly related to dose. Wild-type mice were dosed with 50- or 100 mg/kg olaparib, and PK parameters determined as described in Materials and Methods. Parameter values are tabulated (**A**) and plotted (**B & C**) versus dose.